

H. C. Yen · B. A. Shelton · L. R. Howard · S. Lee
J. Vrebalov · J. J. Giovannoni

The tomato *high-pigment* (*hp*) locus maps to chromosome 2 and influences plastome copy number and fruit quality

Received: 26 June 1997 / Accepted: 22 July 1997

Abstract The tomato (*Lycopersicon esculentum*) *high-pigment* (*hp*) locus was originally described as having enhanced fruit-quality characteristics and has also been shown to regulate responses to light during growth and development. Specifically, the *hp* phenotype suggests that the normal *HP* gene-product serves as a negative regulator of light signal-transduction, as has been proposed for many of the previously described *Arabidopsis thaliana* photomorphogenic mutants. Consequently, *hp* represents a tool for both genetic dissection of light signal-transduction and manipulation of fruit quality in tomato. As a first step toward isolation of the *HP* gene, the *hp* locus was mapped to tomato chromosome 2, adjacent to the 45s rDNA locus, using DNA markers and an interspecific cross of *L. esculentum* × *L. cheesmannii*. We have simultaneously identified DNA markers which may be useful for gene isolation and marker-assisted selection. We have additionally extended characterization of the *hp* phenotype to demonstrate increased sucrose and flavonoid accumulation in ripe *hp/hp* fruit. Analysis of plastid DNA copy number relative to genomic DNA content indicates that the *hp* locus regulates plastome DNA concentration, and possibly plastid number, in response to light.

Key words Photomorphogenesis · Carotenoids · Flavonoids · RFLP mapping

Introduction

The transition from green to red color, characteristic of ripening tomato fruit, is due largely to the transition of chloroplasts to chromoplasts as photosynthetic membranes are disrupted, chlorophyll is degraded, and carotenoids including β -carotene and lycopene accumulate (Grierson 1985). The regulation of carotenoid biosynthesis during ripening has been shown to be due at least in part to ripening-related and ethylene-inducible gene expression (Bird et al. 1991; Gray et al. 1994). While numerous tomato mutants altered in pigment accumulation have been reported (Rick 1980), few which result in enhanced carotenoid accumulation have been identified. Exceptions include the *crimson* (*og^c*) mutation which confers elevated lycopene accumulation at the expense of β -carotene, resulting in dark-red fruit but with little change in levels of total carotenoids (Thompson et al. 1962). In contrast, the recessive *high-pigment* (*hp*) mutation results in increased accumulation of both lycopene and β -carotene during fruit development in addition to heightened levels of chlorophyll in leaves and green fruit (Wann et al. 1985). A second mutation, similar in phenotype to *hp* and termed *hp-2*, was described by Soressi (1975) and has recently been demonstrated to be non-allelic with *hp* (van Tiunen et al. 1997).

In addition to the alteration of fruit and foliar pigmentation, tomato seedlings homozygous for the mutant *hp* allele demonstrate an exaggerated photomorphogenic de-etiolation response (Peters et al. 1989). In short, *hp/hp* seedlings are characterized by the inhibition of hypocotyl elongation and intense anthocyanin pigmentation, relative to seedlings of a normal near-isoline, with maximal phenotypic expression in response to red light (Peters et al. 1992). Tomato seedling de-etiolation is a phytochrome (red-light) response which can additionally be accelerated by blue light, suggesting that *hp* may influence

Communicated by R. Hagemann

H. C. Yen · B. A. Shelton · L. R. Howard¹ · S. Lee
J. Vrebalov · J. J. Giovannoni (✉)

Department of Horticultural Sciences and Texas A and M Vegetable Improvement Center, Texas A and M University, College Station, TX 77843-2133, USA

Fax: 409-845-0627

e-mail: jjg@unix.tamu.edu

Present address:

¹ Department of Food Science, University of Arkansas, Fayetteville AR 72701, USA

phytochrome and blue-light receptor action or signal transduction. Over-expression of oat phytochrome A in tomato resulted in a phenotype similar to that observed in the *hp* mutant (Boylan and Quail 1989). Furthermore, Peters et al. (1992) showed that the *hp* phenotype was repressed when associated with the phytochrome-deficient *aurea* (*au*) mutant, confirming the role of *hp* in phytochrome responses. Quantitation of phytochrome levels in normal and *hp/hp* seedlings indicates that the amplified phytochrome responses observed in the *hp* mutant occur in the context of normal phytochrome concentration and stability, suggesting that the normal *HP* gene product acts as a negative regulator of phytochrome signal-transduction in tomato (Adamse et al. 1989; Peters et al. 1992).

In recent years, the model plant genetic system *Arabidopsis thaliana* has emerged as a valuable source of genetic information for light signal-transduction in plants. While a number of distinct plant photoreceptors have been identified, most wavelengths of light appear to have some overlapping transduction-pathway components in *Arabidopsis* (Chory 1993; Quail 1994). As such, numerous light responses are affected in each of the *det*, *cop*, and *hy* mutants (reviewed in Chory 1993; Deng 1994; Quail 1994; Kowk et al. 1996). The *det* (*de-etiolated*) and *cop* (*constitutively photomorphogenic*) mutants possess normal levels of phytochrome and display normal de-etiolation whether germinated in complete darkness or in the light (Quail et al. 1995). The *hy5* mutant is defective in blue-, red- and far-red-light-controlled responses suggesting a genetic lesion in a signal-transduction component common to all three light regimes and involving multiple photoreceptors (Koornneef et al. 1980). A number of mutants repressed in light response have been shown to represent lesions in the accumulation of specific photoreceptors (*hy3*, *hy4*, *hy8*; Somers et al. 1991; Ahmad and Cashmore 1993; Parks and Quail 1993; Reed et al. 1993), while others represent steps in associated chromophore biosynthesis (*hy1*, *hy2*; Parks and Quail 1991). Nevertheless, the majority of the remaining mutations have been hypothesized to represent genes which function as negative regulators of light signalling in *Arabidopsis* (Chory 1993; Bowler and Chua 1994; Quail 1994; Wei et al. 1994).

A number of genes corresponding to mutant *Arabidopsis* light-signalling loci have been isolated. COP1 protein demonstrates dark-dependent nuclear localization (von Arnim and Deng 1994) and serves as a repressor of photo-morphogenesis (McNeills et al. 1994). COP9 and COP11 are components of a multi-subunit complex which regulates nuclear localization of COP1 in the dark (Chamovitz et al. 1996). DET1 also exhibits nuclear localization but has not been shown to be light-regulated (Pepper et al. 1994). While these interactions may give some insight into the relative regulatory hierarchy among some of the genet-

cally defined light-signalling components, specific mechanisms of light signal-transduction remain poorly understood.

In tomato, red light alone is sufficient to stimulate anthocyanin synthesis and de-etiolation in seedlings, although blue light can also accelerate this process. In *Arabidopsis*, however, both red and blue light are necessary for de-etiolation of seedlings (Koornneef and Kendrick 1994), suggesting: (1) specific mechanisms for light induction of plant developmental process, and/or (2) specific effects or targets of a common regulatory system, which may have diverged among species (Mohr 1994).

Complete characterization of the *hp* phenotype, in addition to eventual isolation and characterization of the corresponding normal and mutant alleles of the *HP* gene, will further our understanding of light signal-transduction in plants and will provide a starting point for comparative analysis of signal-transduction mechanisms, and/or evolved use of conserved mechanisms, among plant species. As initial steps toward the isolation and more complete biochemical and functional characterization of the *HP* gene, we have analyzed fruit-flavonoid and sugar accumulation and have observed significant variations in the presence of *hp*. In addition, plastome DNA copy number increases several fold in the presence of *hp* as compared to nearly isogenic controls.

Finally, we have localized the *hp* locus to tomato chromosome 2 using a combination of RAPD and RFLP markers. Precise chromosomal localization will permit us to test for linkage with tomato homologues of *Arabidopsis* light signal-transduction genes and, additionally, represents the first step in gene isolation based on either map position or heterologous transposon tagging, should *hp* prove to be novel. Eventual isolation of the *HP* gene will facilitate analysis of the mechanisms of light signal-transduction in tomato and permit comparative analysis with additional plant species, including *Arabidopsis*.

Materials and methods

Plant material and phenotypic scoring

Seeds from normal tomato (*Lycopersicon esculentum*) CV Ailsa Craig and a line nearly isogenic and homozygous for the *high-pigment* (*hp*) mutation were obtained from the Glasshouse Crops Research Institute (Littlehampton, UK). Seeds from the wild tomato relative *Lycopersicon cheesmannii* (accession No. LA483) were kindly provided by C. Rick (University of California, Davis, USA). An F₂ population was generated by selfing progeny resulting from a cross between *L. esculentum* (*hp/hp*) and *L. cheesmannii* (*Hp/Hp*). All F₂ seedlings were germinated at 20°C in the dark for 7 days and then transferred to a 12-h white light/12-h dark cycle for the next 7 days. Seedlings were scored as either normal (*Hp/-*; light-colored elongated hypocotyls) or mutant (*hp/hp*; short purple hypocotyls), transplanted to 8-inch pots, and grown to maturity under standard greenhouse conditions. F₃ seed was extracted and germinated, as

above, to both confirm the F_2 phenotype and distinguish Hp/Hp from hp/hp individuals.

Normal "Breaker plus 7 d" fruit are red ripe fruit harvested 7 days after the first signs of color development. Fruits from normal and mutant genotypes were tagged at anthesis, and mutant fruit was harvested at the same time as the corresponding normal fruit of the same age. No significant alterations in time-to-onset or duration-of-ripening were observed between Hp/Hp and hp/hp fruit.

Isolation and hybridization of nucleic acids

Procedures for both genomic DNA extraction from expanding tomato leaves and DNA gel-blot hybridization were performed as described previously (Tanksley et al. 1992; Fulton et al. 1995). Alkaline DNA gel-blotting was as described by the supplier of the nylon membrane employed (Hybond- N^+ , Amersham). High-stringency hybridizations were for 36 h at 65°C in $5 \times$ SSC, 0.5% (w/v) SDS, 50mM Na-P (pH 7.5), and $5 \times$ Denhardt's solution, and were followed by 20-min 65°C washes in first $2 \times$ SSC, 0.1% (w/v) SDS, then $1 \times$ SSC, 0.05% (w/v) SDS, and finally $0.5 \times$ SSC, 0.05% (w/v) SDS. Identification of RFLPs was as described by Tanksley et al. (1992), including additional restriction endonucleases as in Yen et al. (1995). Restriction enzymes yielding RFLPs were used to digest genomic DNA extracted from individual members of segregating populations for subsequent linkage analysis.

Bulked-segregant analysis

Two DNA pools ("mutant" and "normal") nearly isogenic for the hp locus region were constructed from segregating F_2 progeny as described by Giovannoni et al. (1991) except that the target locus itself (as in Michelmore et al. 1991), in addition to flanking RFLP loci, was considered for inclusion in a particular pool. Genomic DNA (5 μ g) from each of six hp/hp F_2 individuals was combined to generate the mutant DNA pool, whereas DNA from four Hp/Hp F_2 individuals was used to generate the normal DNA pool. All pool members were derived from the F_2 population described above. Three-hundred random 10-base primers (Operon Technologies, Alameda, Calif.) were individually tested in the amplification of 10–20 ng of each pooled DNA template under previously described conditions (Giovannoni et al. 1991).

Cloning and amplification of RAPD markers

Linkage of polymorphic RAPD PCR products was verified via amplification of individual pool members as described previously (Giovannoni 1991). DNA bands representing sequences putatively linked to the hp locus were then excised from agarose gels, purified (GeneClean II; Bio 101, Vista, Calif.), and cloned into the PCR-product cloning vector pGEM-T as described by the supplier (Promega, Madison, Wis.). Cloned inserts were amplified using vector-specific primers (M13F, M13R universal primers) and separated from un-incorporated nucleotides via passage through a sephadex G-50 (Sigma, USA) spin column constructed from a 1-ml syringe. Radiolabeling was performed using the random hexamer method as described by Feinberg and Vogelstein (1983).

Genetic linkage analysis

F_2 seed segregating for the hp and RFLP loci was generated from the cross *L. esculentum* (hp/hp) \times *L. cheesmannii* (Hp/Hp). Genetic linkage analysis was performed using MapMaker software (Lander et al. 1987). All RFLP markers used had been localized previously to

tomato chromosomes 2 and 12 (Tanksley et al. 1992). The "group", "compare", and "ripple" commands were utilized, respectively, to verify the order of markers in our cross. Additional RFLPs derived from RAPD markers, and RAPD markers which were not polymorphic as RFLPs and thus scored only as RAPDs, were added to the map using the "try" and "ripple" commands, respectively. CentiMorgan distances were calculated using the Kosambi (1944) function.

Chromosome-2 DNA marker-loci RFLP identification

Twenty one DNA marker loci spanning tomato chromosome 2 were used as hybridization probes to identify RFLPs which could be mapped in the F_2 population segregating for hp . *L. esculentum* (hp/hp) and *L. cheesmannii* (Hp/Hp) genomic DNAs were digested with 35 different restriction endonucleases, separated by agarose-gel electrophoresis, transferred to nylon membranes (Hybond N^+ , Amersham) and hybridized with radiolabeled DNA marker probes. Eleven chromosome-2 markers yielded mappable polymorphisms with the indicated enzymes: namely, CT196 (*EcoRI*), CT205 (*HincII*), CT255 (*EcoRV*), TG165 (*EcoRV*), TG276 (*BstOI*), TG290 (*BstOI*), TG293 (*HaeIII*), TG462 (*BstOI*), TG522 (*EcoRI*), TG582 (*DraI*), 45s rDNA (*DraI*).

Extraction and quantification of fruit flavonoids

Flavonoid glucoside extraction and HPLC analysis was described in Hertog et al. (1992) except that hydrolysis of extracted compounds was modified according to Lee et al. (1995). Known concentrations of purified quercetin, luteolin and kaempferol (Sigma, USA) were used as standards.

Extraction and quantitation of fruit sugars

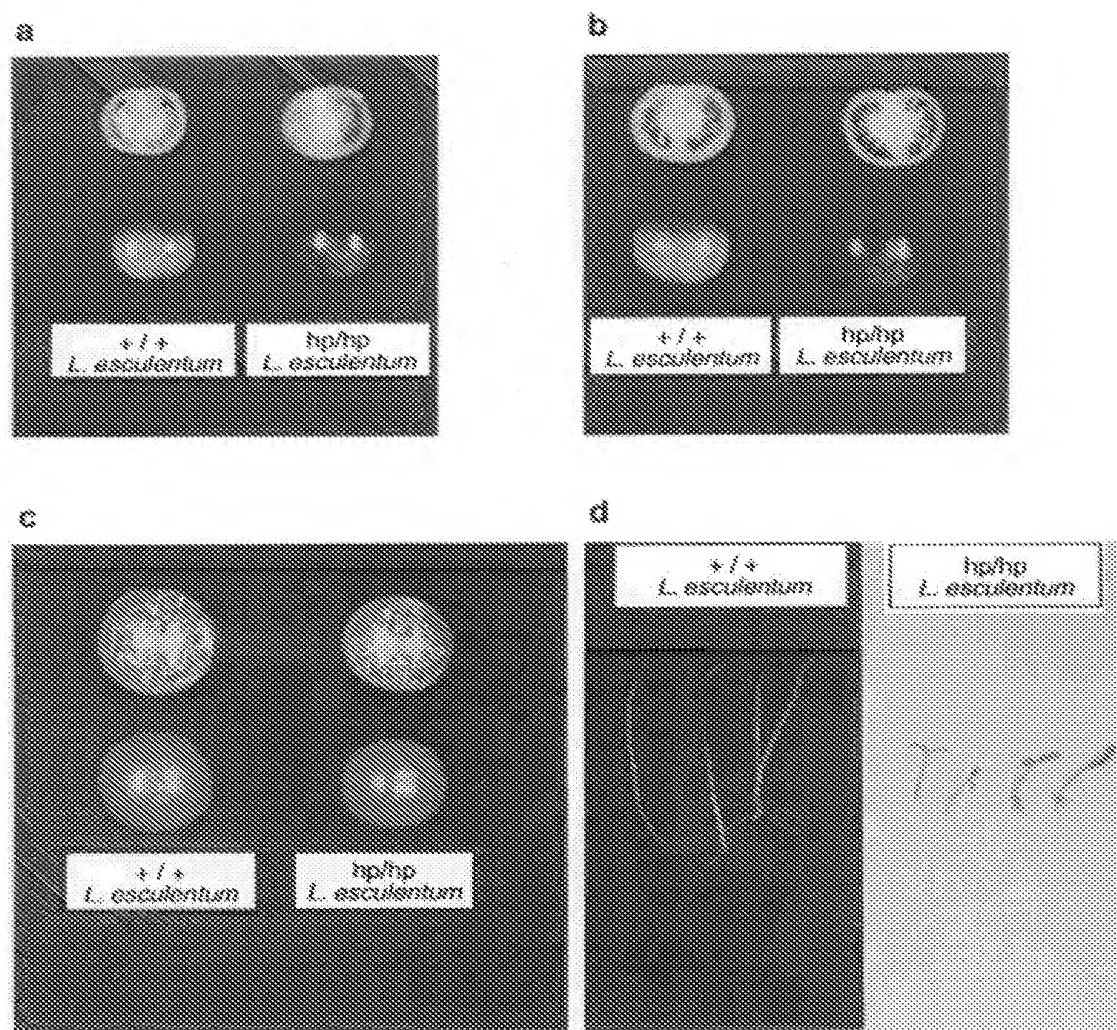
Glucose, fructose, and sucrose extraction and quantitation were as described in Howard et al. (1994) using HPLC and known concentrations of glucose, fructose, and sucrose (Sigma, USA) as standards.

Results

Genetic analysis of a population segregating for hp and DNA marker loci

The hp locus was originally assigned to the short arm of tomato chromosome 12 (Kerr 1979). However, data sufficient for specific localization relative to other morphological markers in this linkage group were not available (Rick 1980). In an attempt to confirm and localize the hp locus, a population segregating for hp and RFLP marker loci was generated. *L. esculentum* (cv Ailsa Craig, hp/hp) and *L. cheesmannii* (accession LA483, Hp/Hp) were crossed to generate F_1 hybrids which were subsequently selfed to create an F_2 population segregating for hp and RFLP loci. Initially, 37 progeny were grown to maturity and F_3 seed was extracted from corresponding ripe F_2 fruit. An additional 1379 F_2 individuals were germinated in the dark at 20°C for 7 days and then transferred to a 12-h light/12-h dark cycle for 7 additional days at 20°C.

Fig. 1a–d Tomato fruit and seedling phenotypes of *L. esculentum* normal (+/+) and mutant (*hp/hp*) individuals of the cultivar Ailsa Craig. (a) Immature green fruit, (b) mature green fruit, (c) red ripe fruit (Breaker + 7 days), (d) seedlings grown 7 days in the dark followed by 7 days in a 12-h light/12-h dark cycle



Seedlings were scored for *hp* phenotype based on the intensity of anthocyanin pigmentation (Mochizuki and Kamimura 1985) as shown in Fig. 1d.

Nine of the original 37 F_2 s were scored as *hp* based on an analysis of F_3 seed (consistent with a single-locus recessive trait: $\chi^2 = 0.0067$; $P > 0.9$). Although we were less confident in direct scoring of F_2 seedlings and observed an error rate of approximately 9% (see below), 371 of the 1379 germinated were scored as *hp* (345 expected). 175 of the 371 putative *hp/hp* F_2 s were grown to maturity resulting in 151 individuals which yielded sufficient seed for F_3 confirmation of the F_2 genotype at the *hp* locus. Twenty F_3 seed from each F_2 individual were germinated for 7 days in the dark followed by 7 days in the light, as described above; 138 *hp/hp* (anthocyanin accumulation in all F_3 seedlings) and 13 *Hp/hp* (segregating 1:1 for anthocyanin accumulation) individuals were identified. Consequently, a total of 188 F_2 progeny, which were genotypically confirmed in the F_3 generation, were used for the construction of a linkage map of the region encompassing the *hp* locus.

Linkage analysis with chromosome-12 markers

Because *hp* was originally assigned to tomato chromosome 12 (Kerr 1979), low- or single-copy RFLP markers spanning the length of this linkage group, and separated on average by 5–20 cM, were selected for initial linkage analysis with *hp* (Tanksley et al. 1992; CT19A, CT79, CT80B, CT211A, TG28, TG68, TG180, TG360, TG394 and TG473). Mappable polymorphisms between the parents of the F_2 population (*L. esculentum* and *L. cheesmannii*) were determined by DNA gel-blot analysis (employing seven restriction endonucleases; *Bgl*III, *Bst*oI, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hind*III). All ten RFLP markers were then scored in a subpopulation of 75 F_2 progeny genotyped for the *hp* locus via the analysis of F_3 seedlings. The relative positions and genetic distances separating chromosome-12 markers were determined as described by Lander et al. (1987) and resulted in an order of loci identical to that reported previously (data not shown; Tanksley et al. 1992). Recombination frequencies were

generally similar, though not identical, to those described by Tanksley et al. (1992) for a F_2 population derived from the cross *L. esculentum* \times *L. pennellii* (data not shown). *hp* did not demonstrate significant linkage to any of the chromosome-12 markers and consequently could not be placed in the chromosome-12 linkage group (data not shown). This result indicated that *hp* is likely to reside in a linkage group other than that corresponding to chromosome-12.

Isolation of RAPD markers linked to *hp*

Bulked-segregant analysis (Michelmore et al. 1991) was performed in an attempt to identify and isolate DNA (RAPD) markers linked to the *hp* locus. Once identified, RAPD markers could then be cloned and utilized as RFLP probes with the mapping population of

Tanksley et al. (1992) to determine linkage to a specific tomato chromosome. Similar approaches have been used to map numerous loci of scientific and agricultural importance, including several involved in tomato fruit development (Giovannoni et al. 1991; Yen et al. 1995).

Genomic DNA from six homozygous *Hp/Hp* (normal) and four homozygous *hp/hp* (mutant) individuals from the *hp* F_2 population was isolated and combined to form "normal" and "mutant" DNA pools, respectively. The "normal" and "mutant" genomic DNA pools were utilized as templates for RAPD amplification as described previously (Giovannoni et al. 1991; Martin et al. 1991). A total of 300 random 10-mers (Operon Technologies) were screened for amplification polymorphisms potentially linked to *hp* (Fig. 2a). Primers which yielded reproducible amplification polymorphisms were employed to amplify genomic DNA derived from individual pool members for confirmation of linkage to *hp* (Fig. 2b). RAPD primers

Fig. 2a, b BSA analysis for the identification of DNA markers linked to *hp*. (a) *hp/hp* (+) and *Hp/Hp* (−) pools were amplified with unique random 10-mers (a–k). The arrow indicates a polymorphism resulting from amplification with primer "a" specific to the *Hp/Hp* pool. We have designated this allele as RAPDhp-11; (b) individual normal and mutant pool members were amplified with primer "a" and only members of the "normal" pool contain the amplifiable RAPDhp-11 allele, suggesting linkage to the *hp* locus

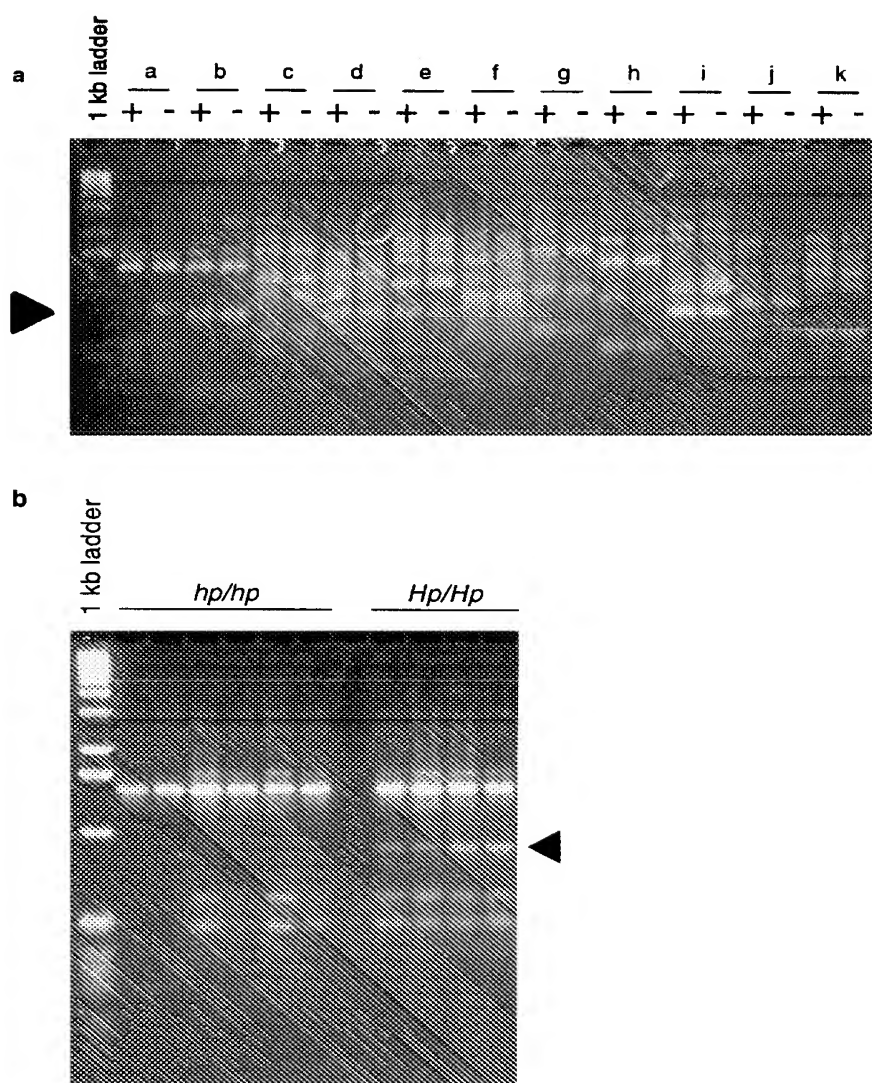


Table 1 Summary of RAPDs and linkage distance from *hp*

Marker	Distance from <i>hp</i>
RAPDhp-7	1.6 cM
RAPDhp-10	4.6 cM
RAPDhp-11	14.6 cM

whose corresponding amplification patterns were consistent with linkage to *hp* in at least 8 of the 10 pool members were then utilized in the amplification of genomic DNA isolated from the remaining 65 F₂ progeny scored for chromosome-12 RFLP markers. A total of five RAPD polymorphisms were scored in the entire subpopulation of 75 F₂s, and three indicated significant linkage to the *hp* locus (Table 1).

The *hp* locus resides on tomato chromosome 2 near the 45s ribosomal repeat

The three RAPD markers linked to the *hp* locus (Table 1) were gel-purified and cloned into the PCR-product cloning vector pGEMT (Promega). DNA gel-blot analysis of *L. esculentum* and *L. cheesmannii* genomic DNA, using the cloned RAPD products as probes, indicated that only RAPDhp-7 is single copy and that RAPDhp-10 and RAPDhp-11 are highly repetitive (data not shown). RAPDhp-7, the marker most tightly linked to *hp*, represents a sequence which is in fact absent, or else highly diverged, from the *L. esculentum* genome (Fig. 3), and could therefore be easily mapped as a dominant marker in both the population segregating for *hp* (*L. esculentum* × *L. cheesmannii*) and the population used to assign chromosomal linkage (*L. esculentum* × *L. pennellii*; Tanksley et al. 1992). Scoring data derived from the use of RAPDhp-7 as an RFLP probe was identical to scoring based on RAPD analysis (Fig. 3).

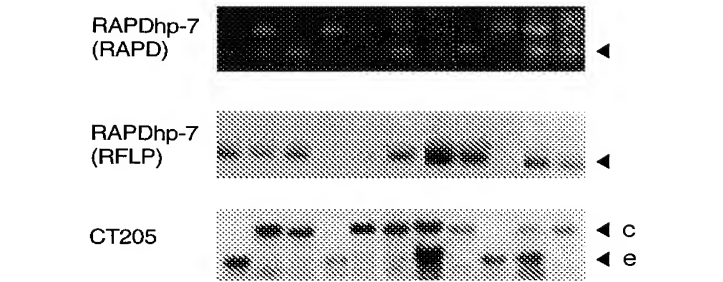


Fig. 3 RAPD and RFLP mapping of RAPDhp-7. RAPD (top) and RFLP (center) segregation in a sample of F₂ progeny from the cross *L. esculentum* (*hp/hp*) × *L. cheesmannii* (*Hp/Hp*). RFLP segregation for CT205, a linked chromosome-2 marker flanking the *hp* locus, is also shown (bottom). Arrows indicate *hp*-linked alleles. “c” and “e” refer to the *L. cheesmannii* and *L. esculentum* alleles of CT205, respectively

Following confirmation of linkage to *hp*, RAPDhp-7 was mapped as an RFLP probe in a subset of the *L. esculentum* × *L. pennellii* F₂ population described by Tanksley et al. (1992), and showed tight linkage to tomato chromosome 2 (Fig. 4). Twenty one RFLP markers spanning chromosome 2 and separated by 5–20 cM were assayed for RFLPs, as described above, and 11 which could be easily scored (see Materials and methods) were mapped in the subpopulation of 188 F₂ progeny. The results are summarized in Fig. 4 and show that the *hp* locus resides in the 6.7 (± 1.3)-cM interval flanked by the 45s ribosomal repeat (45s) and the single-copy marker TG205. The co-segregating 45s and RAPDhp-7 loci are the closest to *hp* at 1.6 (± 0.7)cM. The localization of RAPDhp-10 and RAPDhp-11 in Fig. 4 is based on the scoring of both markers as RAPDs rather than as RFLPs, due to their complex hybridization patterns.

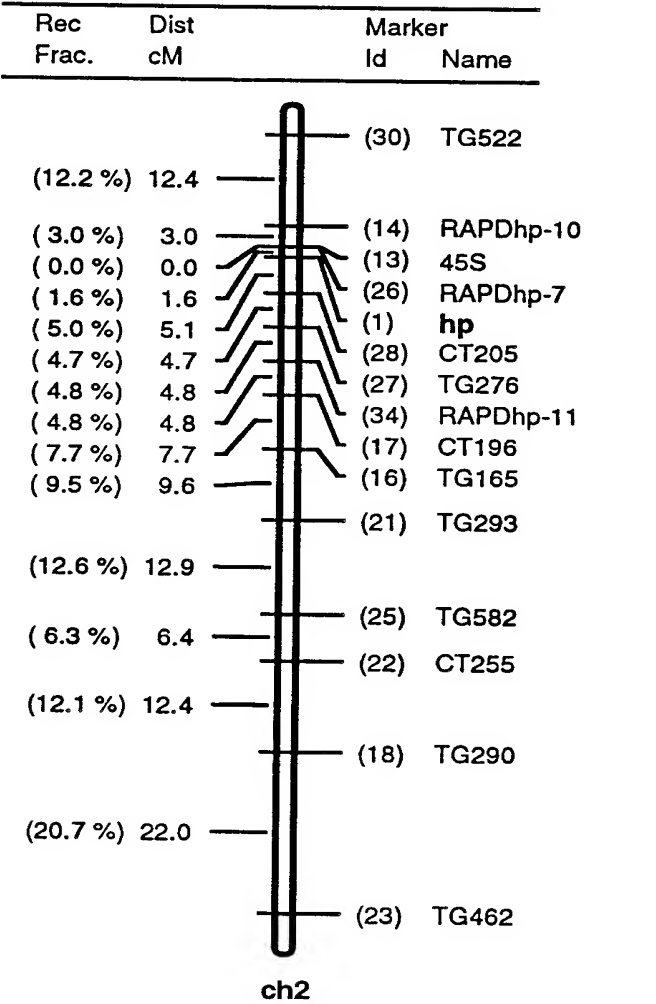


Fig. 4 Linkage map of tomato chromosome 2 resulting from the analysis of F₂ progeny from the cross *L. esculentum* (*hp/hp*) × *L. cheesmannii* (*Hp/Hp*)

In summary, the demonstration of tight genetic linkage to multiple chromosome-2 marker loci provides strong evidence that *hp* is in fact located on chromosome 2 rather than chromosome 12. In addition, the close proximity of *hp* relative to the 45s ribosomal repeat may make this gene a challenging target for isolation based on a genetic map-based cloning strategy.

Analysis of flavonoid accumulation in normal and *hp/hp* fruit

Flavonoids represent a class of related compounds, including flavonones, flavonols, isoflavonoids and anthocyanins, which are unique to plants. All flavonoids share a common precursor, chalcone, which is synthesized through the stepwise condensation of acetate residues derived from three molecules of malonyl-CoA and one molecule of 4-coumaroyl-CoA via the catalytic activity of chalcone synthase (Heller and Hahlbrok 1980). Chalcone synthase gene-transcription has been shown to be influenced by light (reviewed in Batschauer et al. 1994), suggesting that flavonoid biosynthesis may be modified in the *hp* mutant. Seedling hypocotyl anthocyanin accumulation has previously been shown to be increased several fold in *hp/hp* tomatoes as compared to nearly isogenic controls (Peters et al. 1989); however, data pertaining to the accumulation of additional flavonoids in *hp/hp* tomatoes has not been reported.

Fruit from normal (*Hp/Hp*) and nearly isogenic mutant (*hp/hp*) tomatoes (cv Ailsa Craig) were tagged at the breaker stage (first sign of visible lycopene accumulation) and harvested 7 days later when red and fully ripe. Seeds and locular tissue were removed and fruits were combined into pools of 2–4 and frozen at -80°C . Pooled frozen fruits were pulverized, and three pools per genotype were extracted and analyzed for flavonoids as described by Lee et al. (1995) using HPLC and purified flavonoid (quercetin, luteolin, kaempferol) standards.

Significant levels of luteolin and kaempferol were not detected in either normal or mutant ripe fruit (data not shown), consistent with previous reports of tomato fruit-flavonoid accumulation (Crozier et al. 1997). However, quercetin accumulation was elevated over 13-fold from 29.5 ppm in normal tomatoes to 405.9 ppm in *hp* fruit (Fig. 5a).

Analysis of sugar accumulation in normal and *hp/hp* fruit

Others have reported increased chlorophyll and carotenoid concentrations in the *hp/hp* mutant (Baker and Tomes 1964; Jarret et al. 1984; Wann et al. 1985) which may result in altered photosynthetic activity and, in turn, may impact on carbohydrate metabolism and

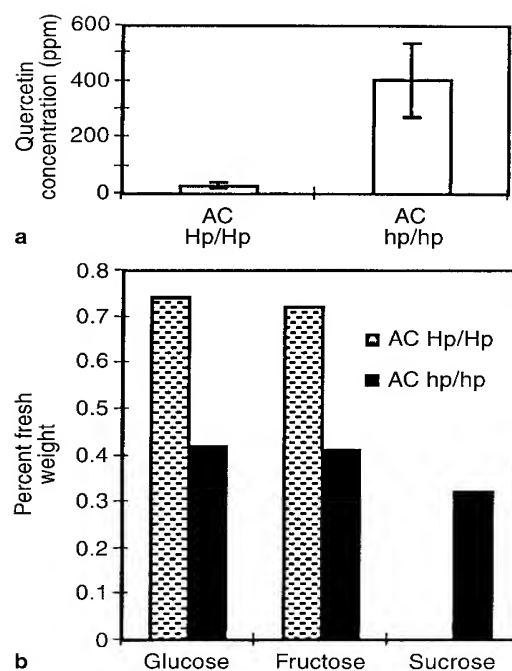


Fig. 5a, b Analysis of flavonoid and sugar concentrations in normal and mutant fruit. (a) Quercetin concentration in the tomato pericarp of nearly isogenic Ailsa Craig *Hp/Hp* and *hp/hp* fruit. (b) Sugar concentrations in the tomato pericarp of nearly isogenic Ailsa Craig *Hp/Hp* and *hp/hp* fruit

ultimate fruit quality. To test this hypothesis, normal and mutant red ripe tomato fruit (breaker + 7 days) were analyzed for the accumulation of glucose, fructose, and sucrose. Figure 5b shows that a dramatic shift in sucrose accumulation is correlated with homozygosity for the mutant allele at the *hp* locus. The shift in sugar accumulation in the *hp* mutant appears to be at the expense of glucose and fructose in that the magnitude of decrease for both is similar to the level of sucrose accumulation (Fig. 5b).

The *hp* mutation results in an increase in the plastome/nuclear genome DNA-content ratio

The increased pigment accumulation in *hp/hp* plants is most likely due to one of the following; (1) an increase in plastid number, (2) an increase in pigment concentration per plastid, (3) a combination of the two. In an attempt to elucidate the mechanism through which the *hp* mutation influences the concentration of photosynthetic pigments, normal and mutant tomato seedlings were germinated for 7 days in the dark followed by 7 days in light. Genomic and organellar DNA were co-isolated from four sets each of normal and mutant hypocotyls via an extraction technique that does not include nuclei enrichment (Fulton et al. 1995). Total DNA was digested with *EcoRI*, separated by agarose-gel

electrophoresis, and blotted to nylon membranes prior to simultaneous hybridization with a single-copy genomic DNA probe (TG503; Tanksley et al. 1992) and a sequence derived from a non-coding region of the plastid genome (Havey 1995). Hypocotyls were chosen as they display the clearest of the *hp* phenotypes (Fig. 1) and were thus hypothesized most likely to show any differences in plastid number and/or organellar DNA content.

Figure 6a shows hybridization to one restriction fragment corresponding to the genomic DNA probe (G), and hybridization to two restriction fragments corresponding to the plastid DNA probe (C1, C2), in both the normal and mutant DNAs. Densitometry was performed on all bands, and ratios of plastid/genomic probe signal intensity within lanes were calculated as an indirect method for calculating the relative amounts

of plastome versus genomic DNA. Mean ratios resulting from a comparison of bands C2 and G are shown in Fig. 6b. Similar results were obtained comparing C1 and G (data not shown).

In summary, hypocotyls sectioned from seedlings which were homozygous for the mutant *hp* allele had approximately five-fold higher plastome to genome ratios as compared to identical tissue extracted from nearly isogenic normal seedlings. This result suggests that the *hp* locus is likely to be involved in the regulation of plastome, and possibly plastid, copy number.

Discussion

The tomato *hp* mutation has been exploited as a genetic tool to both manipulate fruit quality (Darby 1978) and study light signal-transduction in plants (reviewed in Koorneef and Kendrick 1994). We report high-resolution genetic mapping of the *hp* locus resulting in its placement near the 45s rDNA locus of tomato chromosome 2. In addition, more extensive phenotypic analysis of normal and nearly isogenic mutant tomato fruit suggests that the *hp* locus influences aspects of carbohydrate and flavonoid metabolism not previously reported. Finally, our observation of increased plastome/genomic DNA ratios in the *hp/hp* mutant suggests a possible explanation through which the *high-pigment* phenotype may be achieved.

Identification of DNA marker loci tightly linked to *hp*

The *hp* seedling phenotype of increased anthocyanin accumulation (Fig. 1d) was used to score F₂ and F₃ progeny of a cross between *L. esculentum* (*hp/hp*) and *L. cheesmannii* (*Hp/Hp*). Several DNA markers linked to the *hp* locus were identified by screening random primers against nearly isogenic normal and mutant DNA pools generated from this population (Fig. 2). Three RAPD markers (RAPDhp-7, RAPDhp-10, and RAPDhp-11) showed moderate-to-tight linkage to *hp* and one, RAPDhp-7 (Fig. 3), was mappable as a single-copy RFLP probe to chromosome 2 in the F₂ population of Tanksley et al. (1992). RAPDhp-7 maps 1.6 cM from *hp* and was localized to chromosome 2 rather than chromosome 12 as previously reported (Kerr 1979). Linkage to chromosome 2 was additionally supported by mapping 11 chromosome-2 RFLP markers in an F₂ population segregating for *hp* (Fig. 4). As the most tightly linked marker, RAPDhp-7 could serve as a molecular tool to assist introgression of *hp* into breeding and/or production lines and could additionally be used as a starting point in a chromosome walk to, or insertional mutagenesis of, the *hp* locus. The fact that the *L. esculentum* allele of RAPDhp-7 cannot be detected via either PCR or DNA gel-blot hybridization

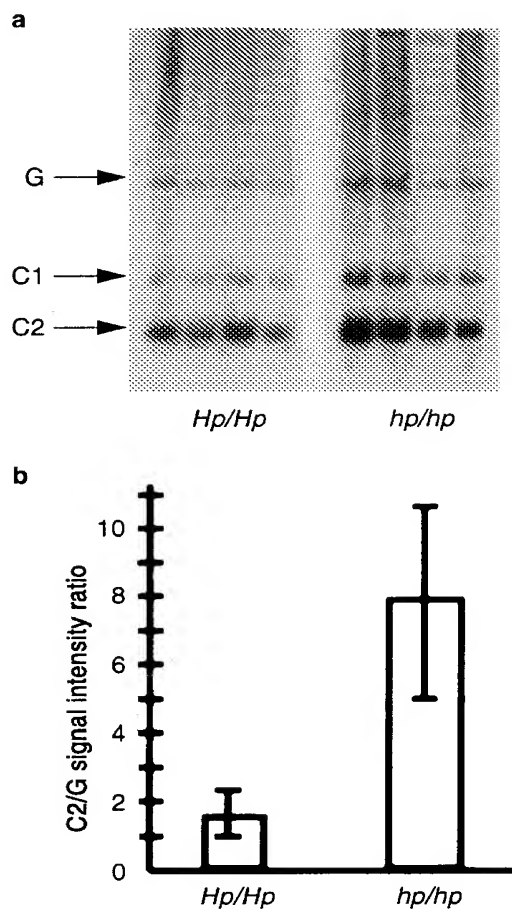


Fig. 6a, b Ratio of plastome to genomic DNA in *Hp/Hp* and *hp/hp* hypocotyls. (a) DNA gel-blot analysis of four individual Ailsa Craig *Hp/Hp* and *hp/hp* plants whose combined genomic and organellar DNA was *Eco*RI-digested and simultaneously probed with a single-copy genomic probe (TG503) and a plastid probe (Havey et al. 1995). "G" is the signal resulting from genomic probe hybridization, while "C1" and "C2" result from hybridization to the plastid probe. (b) Mean C2/G signal intensity as determined by densitometry of the film shown in (a)

would require the use of either *L. cheesmannii* or *L. pennellii* in the development of breeding lines and for library construction, respectively.

A second mutation resulting in a pleiotropic high-pigment phenotype similar to *hp*, and termed *hp-2*, was described by Soressi (1975), and has been suggested to be allelic with *hp* (Mochizuki and Kamimura 1986). *hp-2* has recently been mapped to tomato chromosome 1 (van Tuinen et al. 1997) which, together with our placement of *hp* on chromosome 2, confirms the *hp* and *hp-2* are non-allelic. In addition, van Tuinen et al. (1997) mapped five tomato phytochrome genes relative to RFLP- and morphological-markers, only one of which maps to chromosome 2 (PHYE), in a region, however, distant from *hp*. This result is not surprising as others have previously demonstrated that the *hp* mutation is not associated with alterations in phytochrome content (Adamse et al. 1989). Consequently, the lack of *hp* linkage to known phytochrome loci is consistent with a previously suggested hypothesis in which *hp* regulates a signal-transduction event(s) downstream from phytochrome (Peters et al. 1992).

hp influences the accumulation of flavonoids and sugars in ripe fruit

Increased accumulation of *hp*-seedling anthocyanin, leaf and fruit chlorophyll, and fruit carotenoids has been well documented (Barker and Tomes 1964; Jarret et al. 1984; Wann et al. 1985; Peters et al. 1992) and are readily apparent upon visual inspection (Fig. 1). Increased chlorophyll content and anthocyanin accumulation led us to hypothesize that related compounds may also be influenced by the *hp* locus. To gain insight regarding the effect of *hp* on flavonoid biosynthesis, flavonoids were extracted from identically aged ripe fruit harvested from normal and nearly isogenic mutant tomatoes. In summary, HPLC analysis of fruit flavonoids indicated a 13-fold increase in quercetin concentration (Fig. 5a) with little effect on additional flavonoids (data not shown). The relative increase in *hp/hp* fruit quercetin accumulation is similar to the relative increase in hypocotyl anthocyanin accumulation of 8–10 fold reported previously (Adamse et al. 1989; Peters et al. 1989). The fact that both anthocyanin and quercetin are elevated in *hp/hp* tissues suggests that the *hp* locus is likely to regulate a step(s) of general phenylpropanoid biosynthesis and/or the initial step of committed flavonoid biosynthesis (chalcone synthase) ultimately responsible for the creation of naringenin chalcone, and common precursor of both molecules (Hahlbrock and Scheel 1989). Goud et al. (1991) demonstrated that phenylalanine ammonia lyase (PAL) activity is elevated approximately 3-fold in hypocotyls of *hp/hp* seedlings as compared to normal controls, and thus may provide at least a partial explanation for increased flavonoid accumulation in *hp/hp* tomatoes.

The elevation of levels of photosynthetic pigments in the *hp* mutant were postulated to possibly influence carbohydrate composition, possibly also relating to changes in fruit-quality parameters. Sugars were extracted from red ripe normal and nearly isogenic *hp/hp* tomato fruit of identical age and quantitated via HPLC. *hp* fruit demonstrated a significant accumulation of sucrose as compared to normal controls (Fig. 5b). Additionally, sucrose accumulation appeared to occur at the expense of the two predominant sugars normally found in *L. esculentum* tomato fruit, glucose and fructose. While we did not analyze enzyme activities associated with sucrose metabolism, Wann et al. (1985) reported slightly higher invertase activity in tomato fruit homozygous for *hp*, in contrast to what might be expected based on the observations reported here.

Nevertheless, the fact that *hp* tomato fruits are substantially altered in their sugar accumulation profiles as compared to normal controls suggests the potential for the utilization of the *hp* allele for modification of fruit quality. In addition, the antioxidant-flavonoid quercetin has been reported to inhibit cancer tumor formation in laboratory animals (Verma et al. 1988; National Research Council 1989) suggesting additional fruit-quality enhancement which may be achieved through the introduction of the *hp* allele into commercial tomato varieties.

hp regulates the ratio of plastome to genomic DNA

It is well known that both the plastid number per cell and the number of plastid genomes (plastomes) per plastid can vary significantly both in different tissues and among developmental stages of plants (reviewed in Dale 1988; Mullett 1988). In addition, regulation of plastome-encoded gene expression is influenced by light and can occur at least in part via modification of plastome copy number (Sasaki et al. 1984; Baumgartner et al. 1989; Gruissem and Tonkyn 1993). To address whether the increase in chlorophyll, carotenoids and flavonoids, associated with the *hp* allele, is correlated with general effects of the *hp* locus on plastid or plastome copy number, comparative DNA gel-blot analysis was performed using genomic and plastome-derived sequences.

Hypocotyls from *hp/hp* seedlings demonstrated an increase of approximately 5-fold in plastome DNA relative to genomic-DNA concentration. This result suggests a function for the normal *HP* gene product in modulating plastome copy number in response to light. Preliminary analysis of plastid copy number in tomato hypocotyls suggested an increase in plastid number; however, this increase did not account for the 5-fold increase in plastome DNA (J. Vrebalov and J. Giovannoni, unpublished).

As suggested by others (Adamse et al. 1989; Peters et al. 1992), and supported by our observations of

increased flavonoid accumulation and plastome copy number, *HP* function is consistent with negative regulation of light signal-transduction in tomato. Continued characterization of the *hp* and *hp-2* phenotypes both separately and as double mutants, in addition to eventual isolation and sequencing of their respective normal and mutant alleles, will provide greater insights into the roles of *hp* genes in the regulation of light signal-transduction in tomato, and will additionally permit a comparison of tomato light signal-transduction components to the more extensively characterized *A. thaliana* light signal-transduction system.

Acknowledgements We are grateful to Steve Tanksley for kindly providing tomato RFLP markers. This work was supported by the United States Department of Agriculture (grant # 95-37300-1575 to J.J.G.), the Texas A and M College of Agriculture and Life Sciences Research Enhancement Program (grant # 2-033 to J.J.G. and L.H.), and Zeneca Plant Sciences. H. C. Yen and B. A. Shelton were supported by the Texas Higher Education Coordinating Board (grant # 999902037).

References

- Adamse P, Peters JL, Jaspers PAMP, van Tuinen A, Koornneef M, Kendrick RE (1989) Photocontrol of anthocyanin synthesis in tomato seedlings: a genetic approach. *Photochem Photobiol* 50: 107–111
- Ahmad M, Cashmore AR (1993) The *HY4* gene of *A. thaliana* encodes a protein with characteristics of a blue-light photoreceptor. *Nature* 366: 162–166
- Arnim AG von, Deng XW (1994) Light inactivation of the *Arabidopsis* photo-morphogenic repressor COP1 involves a cell-specific regulation of its nucleo-cytoplasmic partitioning. *Cell* 79: 1035–1045
- Baker LR, Tomes ML (1964) Carotenoids and chlorophylls in two tomato mutants and their hybrids. *Proc Am Soc Hort Sci* 85: 507–513
- Batschauer A, Gilmartin PM, Nagy F, Schafer E (1994) The molecular biology of photoregulated genes. In: Kendrick RE, Kronenberg GHM (eds) *Photomorphogenesis in plants*. Kluwer Academic Publishers, The Netherlands pp 559–599
- Baumgartner BJ, Rapp JC, Mullet JE (1989) Plastid transcription activity and DNA copy number increases early in barely chloroplast development. *Plant Physiol* 89: 1011–1018
- Bird C, Ray J, Fletcher J, Boniwell J, Bird A, Teulieres C, Blain, I, Bramley P, Schuch W (1991) Using antisense RNA to study gene function: inhibition of carotenoid biosynthesis in transgenic tomatoes. *Bio/Technology* 9: 635–639
- Bowler C, Chua NH (1994) Emerging themes of plant signal-transduction. *The Plant Cell* 6: 1529–1541
- Boylan MT, Quail PH (1989) Oat phytochrome is biologically active in transgenic tomatoes. *Plant Cell* 1: 765–773
- Chamovitz DA, Ning W, Osterlund MT, von Arnim AG, Staub JM, Matsui M, Deng XW (1996) The COP9 complex, a novel multi-subunit nuclear regulator involved in light control of a plant developmental switch. *Cell* 86: 115–121
- Chory J (1993) Out of darkness: mutants reveal pathways controlling light-regulated development in plants. *Trends Genet* 9: 167–172
- Crozier A, Lean ME, McDonald, MS, Black C (1997) Quantitative analysis of the flavonoid content of commercial tomatoes, onions, lettuce, and celery. *J Agric Food Chem* 45: 590–595
- Dale JE (1988) The control of leaf expansion. *Annu Rev Plant Physiol Plant Mol Biol* 39: 267–295
- Darby LA (1978) Isogenic lines of tomato fruit-colour mutants. *Hort Res* 18: 73–84
- Deng XW (1994) Fresh view of light signal-transduction in plants. *Cell* 76: 423–426
- Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 132: 6–13
- Fulton T, Julapark C, Tanksley S (1995) Miniprep protocol for extraction of DNA from tomato and other herbaceous plants. *Plant Mol Biol Rep* 13: 207–209
- Giovannoni JJ, Wing RA, Ganai MW, Tanksley SD (1991) Isolation of molecular markers from specific chromosomal intervals using DNA pools from existing mapping populations. *Nucleic Acids Res* 19: 6553–6558
- Gray JE, Picton S, Giovannoni JJ, Grierson D (1994) The use of transgenic and naturally occurring mutants to understand and manipulate tomato fruit ripening. *Plant, Cell and Environment* 17: 557–571
- Grierson D (1985) Gene expression in ripening tomato fruit. *CRC Press Crit Rev Plant Sci* 3: 113–132
- Gruissem W, Tonkyn JC (1993) Control mechanisms of plastid gene expression. *CRC Crit Rev Plant Sci* 12: 19–55
- Goud VK, Sharma R, Kendrick RE, Furuya M (1991) Photoregulation of phenylalanine ammonia lyase is not correlated with anthocyanin induction in photomorphogenic mutants of tomato (*Lycopersicon esculentum*). *Plant Cell Physiol* 32: 1251–1258
- Hahlbrock K, Scheel D (1989) Physiology and molecular biology of phenylpropanoid metabolism. *Annu Rev Plant Physiol Plant Mol Biol* 40: 347–369
- Havey MJ (1995) Identification of cytoplasmic using the polymerase chain reaction to aid in the extraction of maintainer lines from open-pollinated populations of onion. *Theor Appl Genet* 90: 263–268
- Heller W, Hahlbrock K (1980) Highly purified “flavone synthase” from parsley catalyzes the formation of naringenin chalcone. *Arch Biochem Biophys* 200: 617–619
- Hertog MLG, Hollman PCH, Venema DP (1992) Optimization of quantitative HPLC determination of potentially anticarcinogenic flavonoids in fruit and vegetables. *J Agric Food Chem* 40: 1591–1598
- Howard JR, Yoo KS, Pike LM, Miller GH (1994) Quality changes in diced onions stored in film packages. *J Food Sci* 59: 110–114
- Jarret RL, Sayama H, Tigchelaar EC (1984) Pleiotropic effects associated with the chlorophyll intensifier mutations *high pigment* and *dark green* in tomato. *J Am Soc Hort Sci* 109: 873–878
- Kerr EA (1979) High-pigment, (*hp*), is on chromosome 12. *TGC Rpt* 29: 26
- Koornneef M, Kendrick RE (1994) Photomorphogenic mutants of higher plants. In: Kendrick RE, Kronenberg GHM (eds) *Photomorphogenesis in plants*. Kluwer Academic Publishers, The Netherlands pp 601–630
- Koornneef M, Rolff E, Spruit CJP (1980) Genetic control of light-inhibited hypocotyl elongation in *Arabidopsis thaliana* (L.) Heynth. *Z Pflanzenphysiol* 100: 147–160
- Kosambi DD (1944) The estimation of map distances from recombination values. *Ann Eugen* 12: 172–175
- Kwok SF, Piekos B, Misera S, Deng XW (1996) A complement of ten essential and pleiotropic *Arabidopsis* *COP/DET/FUS* genes is necessary for repression of photo-morphogenesis in darkness. *Plant Physiol* 110: 731–742
- Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. *Genomics* 1: 174–181
- Lee Y, Howard JR, Villalon B (1995) Flavonoids and antioxidant activity of fresh pepper (*Capsicum annuum*) cultivars. *J Food Sci* 60: 473–476
- Martin G, Williams J, Tanksley S (1991) Rapid identification of markers linked to a *Pseudomonas* resistance gene in tomato using random primers and near-isogenic lines. *Proc Natl Acad Sci USA* 88: 2236–2240

- McNellis TW, von Arnim AG, Deng XW (1994) Over-expression of *Arabidopsis* COP1 results in partial suppression of light-mediated development: evidence for a light-inactivable repressor of photo-morphogenesis. *The Plant Cell* 6:1391–1400
- Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. *Proc Natl Acad Sci USA* 88:9828–9832
- Mochizuki T, Kamimura S (1985) Photo-selective method for selection of *hp* at the cotyledon stage. *TGC Rpt* 35:12–13
- Mohr H (1994) Co-action between pigment systems. In: Kendrick RE, Kronenberg GHM (eds) *Photo-morphogenesis in plants*. Kluwer Academic Publishers, The Netherlands, pp 353–373
- Mullet JE (1988) Chloroplast development and gene expression. *Annu Rev Plant Physiol Plant Mol Biol* 39:475–502
- National Research Council (US) Committee on Diet and Health, National Academy of Sciences (1989) *Diet and health: implications for reducing diet and disease risk*. National Academy Press, Washington, D.C.
- Parks BM, Quail PH (1991) Phytochrome-deficient *hy1* and *hy2* long-hypocotyl mutants of *Arabidopsis* are defective in phytochrome chromophore biosynthesis. *The Plant Cell* 3:1177–1186
- Parks BM, Quail PH (1993) *hy8*, a new class of *Arabidopsis* long-hypocotyl mutants deficient in functional phytochrome A. *The Plant Cell* 5:39–48
- Pepper A, Delaney T, Washburn T, Poole D, Chory J (1994) DET1, a negative regulator of light-mediated development and gene expression in *Arabidopsis*, encodes a novel nuclear-localized protein. *Cell* 78:109–116
- Peters JL, van Tuinen A, Adamse P, Kendrick RE, Koornneef M (1989) High-pigment mutants of tomato exhibit high sensitivity for phytochrome action. *J Plant Physiol* 134:661–666
- Peters JL, Schreuder MEL, Verduin SJW, Kendrick RE (1992) Physiological characterization of a high-pigment mutant of tomato. *Photochem Photobiol* 56:75–82
- Quail PH (1994) Photosensory perception and signal transduction in plants. *Curr Opin Genet Dev* 4:652–661
- Quail PH, Boylan MT, Short TW, Xu Y, Wagner D (1995) Phytochrome: Photosensory perception and signal transduction. *Science* 268:675–680
- Reed JW, Nagpal P, Poole DS, Furuya M, Chory J (1993) Mutations in the gene for the red/far-red light-receptor phytochrome B alter cell elongation and physiological responses throughout *Arabidopsis* development. *The Plant Cell* 5:147–157
- Rick CM (1980) Tomato linkage survey. *TGC Rpt* 30:2–17
- Sasaki Y, Tomoda Y, Kamikubo T (1984) Light regulates the gene expression of ribulose biphosphate carboxylase at the levels of transcription and gene dosage in greening pea leaves. *FEBS Lett* 196:171–174
- Somers DE, Sharrock RA, Tepperman JM, Quail PH (1991) The *hy3* long-hypocotyl mutant of *Arabidopsis* is deficient in phytochrome B. *The Plant Cell* 3:1236–1274
- Soressi GP (1975) New spontaneous or chemically induced fruit-ripening tomato mutants. *Tomato Genet Coop Rep* 25:21–22
- Tanksley SD, Ganal MW, Prince, JP, de Vicente MC, Bonierbale MW, Broun P, Fulton TM, Giovannoni JJ, Grandillo S, Martin GB, Messeguer R, Miller JC, Miller L, Paterson AH, Pineda O, Röder MS, Wing RA, Wu W, Young ND (1992) High-density molecular linkage maps of the tomato and potato genomes. *Genetics* 132:1141–1160
- Thompson AE, Hepler RW, Kerr EA (1962) Clarification of the inheritance of high total carotenoid pigments in the tomato. *Proc Am Soc Hort Sci* 81:434–442
- Tuinen A van, Cordonnier-Pratt MM, Pratt LH, Verkerk R, Zabel P, Koornneef (1997) The mapping of phytochrome genes and photo-morphogenic mutants of tomato. *Theor Appl Genet* 94:115–122
- Verma AK, Johnson JA, Gould MN, Tanner MA (1988) Inhibition of 7,12-dimethylbenz(α)anthracene and N-nitro-methylurea-induced rat mammary cancer by the dietary flavanol quercetin. *Cancer Res* 48:5754–5760
- Wann EV, Jourdain EL, Pressey R, Lyon BG (1985) Effect of mutant genotypes *hp og^c* and *dg og^c* on tomato fruit quality. *J Am Soc Hort Sci* 110:212–215
- Wei N, Kwok SF, von Arnim AG, Lee A, McNellis TW, Piekos B, Deng XW (1994) *Arabidopsis* *COP8*, *COP10*, and *COP11* genes are involved in repression of photo-morphogenic development in darkness. *The Plant Cell* 6:629–643
- Yen HC, Lee S, Tanksley SD, Lanahan MB, Klee HJ, Giovannoni JJ (1995) The tomato *Never-ripe* locus regulates ethylene-inducible gene expression and is linked to a homolog of the *Arabidopsis* *ETR1* gene. *Plant Physiol* 107:1343–1353